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Research Article

Acetylcholinesterase, Alpha-Glucosidase and Tyrosinase Inhibitors from Egyptian Propolis

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ABSTRACT

Bioassay guided fractionation led to the isolation of eight compounds from Egyptian Propolis. Hexacosanoic acid; 3,4-dimethoxy-cinnamic acid; 3-methyl-3-butenyl-*trans*-caffeate ester; together with four flavonoids (Chrysin; Pinostrobin; Tectochrysin and 5-hydroxy-7-methoxy isoflavone). 2,3-dihydroxy-4-methyl-octanoic acid; was isolated for the first time from Egyptian propolis. Their acetylcholinesterase (AChE), alpha-glucosidase and tyrosinase inhibition potential were evaluated. Pinostorbin showed the highest AChE inhibitory activity followed by 3,4-Dimethoxycinnamic acid; 3-methyl-3-butenyl-*trans*-caffeate ester. Tectochrysin is the only compound had alpha-glucosidase inhibitory activity higher than that of the drug acarabose. Pinostrobin is the only compound had mild tyrosinase inhibitory activity.

Keywords: Egyptian Propolis, Acetylcholinesterase, alpha-glucosidase, tyrosinase Inhibitors.

INTRODUCTION

Cell aging is a prominent factor that causes irregular functions of many systems; leading to various chronic diseases such as cardiovascular diseases, cancer, metabolic diseases as Type 2 Diabetes Mellitus "T2DM" and neurodegenerative diseases as Alzheimer's disease (AD)[1]. Acetylcholine deficiency in the brain is the main cause of AD in elder patients. Acetylcholinesterase (AChE) activity is an exponent to cell aging in elders[2]. AChE inhibitors are the main target for treating AD. Few synthetic drugs are available which have been reported to have a short half-life as well as many others side effects like hepatotoxicity[3,4]. Finding new natural AD inhibitors is important.

Oxidative damage is one of the earliest causes of AD; antioxidants could be useful in both prophylactic and in treatment[5]. Natural products are considered as important sources of bioactive metabolites for new drugs treating aging diseases[6].

Increase in the prevalence of metabolic diseases as Type 2 Diabetes Mellitus (T2DM) in old age may be related directly with aging process itself or indirectly through several other age-related risk factors of (T2DM) as mitochondrial dysfunction and central obesity, also impaired fasting glycemia (IFG) and Type 2 Diabetes mellitus (T2DM) increase with advanced age[7]. DM is characterized by abnormally high plasma glucose level (Hyperglycemia), the elevated blood glucose level causes significant fluxes of glucose in tissues such as nerves, retina, lens, and kidneys[8], resulted in pathogenesis complications as neuropathy, nephropathy, retinopathy, cataract, atherosclerosis, and impaired wound healing[9]. In treatment of (T2DM), suppression of postprandial hyperglycemia can decrease the risk of those complications. Therefore, control of postprandial hyperglycemia should be the first target to prevent and manage (T2DM). One of the therapeutic approaches for decreasing postprandial hyperglycemia in Diabetic patients is preventing the absorption of carbohydrates after food uptake. Only monosaccharide, such as glucose could be absorbed[10].

If α-glycosidases are inhibited, the liberation of D-glucose from dietary complex carbohydrates can be retarded. Thus, α-glucosidase inhibitors play an important role in delaying the digestion and absorption of carbohydrates and hinder postprandial hyperglycemic deviation.

Currently prescribed synthetic α-glucosidase inhibitor drugs as miglito and acarbose are mainly associated with many unfavorable side effect as diarrhea, flatulence and abdominal pain which annoy DM patients as the drugs are prescribed for long term administration[11]. As a result, many researchers have focused on natural products inhibiting α-glucosidase activity; especially those rich in polyphenols compounds[12], as many natural products are mostly free from bad adverse effects and have excellent pharmacological activities so they could mainly result in development of new safer anti-diabetic agents or diabetic complication resolving agents[13].

Skin aging is a biologic process that results from actual aging and progressive environmental stress. Recent

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researches suggest that cumulative oxidative damage to DNA is due to the continuous generation of free radicals, environmental pollution and increase in free radicles level, could be the first cause in DNA damage.

Aging process is associated with numerous pigmentary disorders, Melanin may increase cause acquired skin hyper-pigmentation (e.g., solar lentigo)[14].

Tyrosinase is important in melanin biosynthesis; the main cause for acquired skin hyper-pigmentation in mammals. So tyrosinase inhibitors can inhibit melanin biosynthesis. It can be so useful clinically for the treatment of many dermatological problems associated with melanin hyper-pigmentation; these encouraged researchers to seek for new natural potent tyrosinase inhibitors with antioxidant power to have dual action[15]**.**

Propolis (bee glue) is a colored and aromatic colloidal substance collected by bees from resins that they collect from different plant organs and then mix it with their secreted saliva; bees used propolis in building honeycomb and in keeping the hive sterile and free of microbial invaders. The chemical composition of propolis varies and depends mainly on the abundance and diversity of plant species visited by bees in the region of its production[16,17] but mainly propolis bioactive chemical compounds are reported to be flavonoids, cinnamic acid derivatives and terpenes[18]. Propolis is well known to possess a broad spectrum of biological activities as anti-inflammatory[19], antibacterial[20], antiviral[20,21] antifungal[22] antioxidant[23] and anticancer activities[24,25].

In our continued search for biologically active compounds from propolis; the present study aimed to evaluate the inhibitory activities of some isolated compounds from Egyptian Propolis to the acetylcholinesterase, alpha-glucosidase and tyrosinase enzymes.

MATERIALS AND METHODS

General

NMR spectra were measured on a Bruker DRX 400 spectrometer (Bruker Biospin, Rhe- instetten, Germany). ^{1}H- and ^{13}C-NMR and HMBC spectra were measured using an inv-erse-detection probe (5 mm). Operating frequencies were 400.13 MHz for acquiring ^{1}H-NMR and 100.6 MHz for ^{13}C-NMR spectra. Samples were measured at 300 K with TMS as an internal standard.

The materials were subjected to chromatographic analysis with High-Performance liquid Chromatography (HPLC); Shimadzu SCL-10Avp System controller, Dual pump shimadzu liquid chromatography (LC-10Avp), shimadzu degasser (DGU-14A), shimadzu UV-Vis detector (SPD-10Avp).

Propolis extraction

Propolis (2 Kg.) was cut into small pieces and extracted with distilled water (2Lx 3) each for 2 hours at 85 °C to give propolis water extract, the residue was extracted with 70% ethanol (2L x 3) under reflux conditions each for 2 h which gave (PEE 70%), this extract was filtered and dried under reduced pressure, the dried residue (70 g.) was suspended in water and then partitioned successively in turn with pet. ether, ether and ethyl acetate.

Ether fraction (35g.) was subjected to Sephadex LH-20 column chromatography (60 x 5 cm) and stepwise gradient elution was carried out using a solvent system of decreasing polarity starting with 100% distilled water then water–methanol. Fractions of 100 ml were collected and investigated by TLC (silica gel DF245 Merck) using different spraying reagents, similar fractions were combined and concentrated to dryness under reduced pressure to obtain one main fraction (27g.) it was fractionated again into many sub-fractions on column packed with silica gel (0.06-0.2mesh, Merck), stepwise elution with petroleum ether, (pet. ether-ethyl acetate) and ethyl acetate was carried out resulted in total nine main sub-fractions.

The ethyl acetate fraction (15 g.) also was further subjected to column chromatography packed with silica gel (0.06-0.2mesh, Merck), elution was carried out with pet.ether, pet.ether-ethyl acetate (9:0.5, 9:1, and 8:2), resulted in total four main sub-fractions.

Bioassay guided fractionation

Bioassay guided fractionation to ether nine main sub-fractions and ethyl acetate four main sub-fractions were carried out by measuring their inhibitory activies against acetylcholinesterase, α-Glucosidase and Tyrosinase enzymes) resulted in five main subfractions (ether sub-fraction no. 4, 6, 8 and ethyl acetate sub-fraction no.2, 4) ; each of these five fractions was chemically investigated by GC/MS and HPLC techniques

Isolated Compounds

The Bioactive subfractions (ether sub-fraction no. 4, 6, 8 and ethyl acetate sub-fraction no. 4); each of these subfractions was rechromatogramed as following resulting in isolation of eight compounds.

The ether sub-fraction no. 4 (12 g.) which showed (AChE) inhibitory activity, was subjected to Sephadex LH-20 column eluted with $CHCl_3$: methanol gradients (9:1; 1:1 and 100% MeOH v/v) led to the isolation of compounds 1& 2 (20, 16.5 mg) respectively.

Ether sub-fraction no. 6 (10 g.) which showed α-glucosidase inhibitory activity, was further fractionated on Sephadex LH-20 column eluted with water: methanol gradients (9:1-1:1, v/v) led to the isolation of Compounds **3** (9 mg.).

The ether sub-fraction no.8 (2.5 g.) which showed also Acetylcholinesterase (AChE) and tyrosinase inhibitory activity, was subjected to Sephadex LH-20 column eluted with $CHCl_3$: methanol gradients (9:1-1:1, v/v,) gave:

One main sub-fraction (120 mg) which needed further purification by preparative normal-phase HPLC [Shimadzu PNP-ODS (H) 250×20mm column , Isocratic elution was carried out all over the run with solvent $CHCl_3$ / MeOH (9:0.5), flow rate 2ml/min and injection volume 50 µl for each run at ambient temperature for 35 minutes yielded compound no.4 (5 mg.) . The compound was detected with a UV detector and the chromatograms were recorded at 320 nm.

One Semipure compound which need recrystalization to give pure compound (no.5 = 8 mg).

Ethyl acetate sub-fraction no. 4 (4.5g.) which possessed also Acetylcholinesterase (AChE) inhibitory activity, was further fractionated on Sephadex LH-20 column, eluted with water:methanol gradients (9:1-1:1, v/v,) led to:
one main sub-fraction (150 mg.) which needed further purification by preparative reversed-phase HPLC [Shimadzu PREP-ODS (H) 250×20mm column] eluted with a gradient elution of water/formic acid (19:1 v/v; solvent A) and acetonitrile (solvent B), started with 20% B, reaches 25% B at 25 min and 30% B at 35 min, and then the system became isocratic until 50 min, reaches 50% B at 60 min and 70% B at 67 min and the flow rate was 1 ml/min, detected by UV detector at 340 nm and yielded compounds no.6 & 7 (5,6 mg. respectively) .
Isolation of One Semipure compound which need recrystalization to give pure compound 8 (18 mg) (Figure 1).

Hexacosanoic acid (1): M^+ [396], ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.3 (2H, t, H-2), δ 1.2 **:** 1.56 [46 H, m, (CH_2-3**:**25)], δ 0.8 (3H,t,H-26). **^{13}**C-NMR: δ 177.3 (C=O), 33.95 (C-2), 31.93 (C-24), 29.7(C-6: C-22), 29.37(C-23), 29.25(C-5), 29.07(C-4), 24.8(C-3), 22.7(C-25), 14.12(C-26).

Chrysin (2): **^{1}**H-NMR (400 MHz, DMSOd6) exhibited a flavonoid pattern and showed signals at δ 12.83(1H, s, 5-OH), δ 10.92(1H, s, 7-OH), 8.07(2H,br.*d, J* = 6.7 ,H-2`,H-6`), δ 7.6 (3H, m, H-3`,H-4`, H-5`) , δ 6.9 (1H, s, H-3), δ 6.5 (1H ,*d* , *J* = 2.04 Hz, H-8), δ 6.2(1H,*d, J* = 2.04 Hz, H-6). Data are in agreement with previous studies[26]

3,4-Dimethoxy-cinnamic acid (3): ^{1}H NMR (400 MHz, DMSOd6)**:** δ 7.5 (1H, *d, J* = 15.9 Hz, H-7), 7.3 (1H, br. *s*, H-2), 7.2(1H, br. *d, J*=8.2 Hz, H-6), 6.99(1H, *d, J*=8.2 Hz, H-5), 6.4 (1H, *d, J* = 15.9 Hz, H-8), 3.8 (6H, *s*, OCH_3-3).**^{13}**C-NMR : δ168.3 (C-9), δ151.2(C-3), δ149.4 (C-4), δ144.6(C-7), δ130.4 (C-1), δ123.11(C-6), δ117.15(C-8), δ114.8(C-5), δ112.00 (C-2), δ56.05(OCH_3). Data are in agreement with previous studies [27]

2,3-Dihydroxy-4-methyl-octanoic acid (4): ^{1}H NMR (400 MHz, DMSOd6): δ 4.4 (1H, d, *J* = 6.4 Hz, H-2); 3.8 (2H, br.s,OH-2, OH-3); 3.13(1H, dd, H-3);1.82(1H, m, H-4); 1.11 (6H, t, CH_2- 5,6,7); 0.82:0.92 (6H, m, CH3-at C-9,C-5).^{13}C-NMR : δ176.55(C-1), δ77.79(C-2), δ70.7 (C-3), δ33.9(C-4), δ28.8(C-5), δ24.01(C-6), δ21.97(C-7), δ19.32(C-8), δ19.24 (C-9).

3-Methyl-3-butenyl-*trans*-caffeate ester (5): ^{1}H NMR (400 MHz, DMSOd6): δ 7. 46 (1H, *d, J*=15.7, H-7), δ 7.04 (1H, *s*, H-2), δ 6.76 (1H, *d, J* = 8.08, H-6), δ 6.99 (1H, *d, J* = 8.12, H-5), δ 6.2 (1H, *d,* J=15.7, H-8), δ 4.19 (2H, *t,* H-1'), δ 4.63 (1H, *d, J* = 7.5, H-4'a), δ 4.79 (1H, *d, J* = 7.5, H-4'b),δ 2.3 (2H, *t,* H-2'), δ 1.74 (3H, *s,* CH_3), **^{13}C-NMR**: δ 167.5 (C=O), δ 126.9 (C-1), δ 114.4 (C-2), δ 148.85 (C-3), δ 148.82 (C-4), δ 115.03 (C-5), δ 121.98 (C-6), δ 145.6 (C-7), δ 116.19 (C-8), δ 62.33 (C-1'), δ 36.78 (C-2'), δ 142.27 (C-3'), δ 112.68 (C-4'), δ 25.86 (C-5'). **HMBC** long-range correlations were observed between the following protons and carbons H-1'at δ 4.19 and (C2' at δ 36.8; C3' at δ 142.27; C9 at δ 167.5); H-2'at δ 2.3 and (C1' at δ 62.33; C3' at δ 142.27; C4' at δ 112.68; C5' at δ 25.86;); H -4' a at δ 4.6 (C3' at δ 142.27; C4' at δ 112.68). Data are in agreement with previous studies [28]

Tectochrysin (6): **^{1}**H-NMR (DMSO d6) exhibited a flavonoid pattern and showed signals at δ 12.8(1H, s, 5-OH), δ 8.13(2H, br. *d, J* = 6.7 ,H-2`,H-6`), δ 7.62 (3H, m, H-3`, H-4`, H-5`) , δ 7.06 (1H, s, H-3), δ 6.8 (1H, *d , J* = 2.16 Hz, H-8), δ 6.4 (1H, *d, J* = 2.12 Hz, H-8), δ3.89 (3H, s,7-OMe). **$^{13}$**C-NMR : δ174.76 (C-4), δ 166.5(C-2), δ 165.86 (C-7), δ 164.1(C-5), δ 161.1 (C-9), δ 132.6 (C-1'), δ 129.65 (C-3`, C-4',C-5`), δ 126.96 (C-2`, C-6`), δ 105.99 (C-10), δ 104.5 (C-3), δ 98.6(C-6), δ 93.3 (C-8), δ 56.61 (C-7-OMe). Data are in agreement with previous studies [26].

5-Hydroxy-7-methoxy isoflavone (7): **^{1}**H-NMR (400 MHz, DMSOd6) exhibited a flavonoid pattern and showed signals at δ 12.82 (1H, s, 5-OH), δ 8.3 (1H, s, H-2) , 8.12 (2H,br.*d, J* = 6.96 ,H-2`,H-6`), δ 7.62 (3H, m, H-3`,H-4`, H-5`) δ 6.8 (1H ,*d , J* = 2.04 Hz, H-8), δ 6.4 (1H,*d, J* = 2.04 Hz, H-8, δ3.89 (3H, s,7-OMe). **$^{13}$**C-NMR δ 181. 06 (C-4), δ165.86 (C-7), δ165.3 (C-5), δ158.29 (C-9), δ 129.65 (C-3`, C-5`), δ128.7 (C-4`), δ 126.96 (C-2`, C-6`), δ 124.21(C-3), δ 105.99 (C-10), δ 99.98 (C-6), δ 94.9 (C-8), δ 56.61 (C-7-OMe). Data are in agreement with previous studies[26].

Pinostrobin (8) : 1H-NMR (400 MHz, DMSO d6) exhibited a flavonoid pattern and showed signals at δ 12.1(1H, s, 5-OH),7.54(2H,br.*d, J* = 7.08 ,H-2`,H-6`), δ 7.42 (3H, m, H-3`,H-4`, H-5`) ,δ 6.16 (1H ,*d , J* = 2.2 Hz, H-8), δ 6.11 (1H,*d, J* = 2.2 Hz, H-8), δ5.63 (1H, *dd, J* =1H, *dd, J* =12.8,2.7, Hz, H-2 Hz, H-2), δ 3.8 (3H, s,7-OMe), δ 3.2 (1H, *dd, J*=1H, *dd, J*=15.1,12.8 Hz, H-3a), δ 2.85 (1H, *dd, J*=14.6, 2.7 Hz, H-3b). Data are in agreement with previous studies[29].

Acetylcholinesterase (AChE) inhibition assay

AChE activity was measured by adapting the colorimetric assay described by Ellman *et al*.[30] with slight modification. Electric-eel AChE (Sigma) was utilized as source of cholinesterase. Acetylthiocholine iodide (Sigma) was used as substrate for AChE, to perform the reaction. 5,5-Dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma) was utilized for the determination of cholinesterase assay. Investigated samples were solubilized in ethanol. Reaction mixture contained 150 µL of (100 mM) sodium phosphate buffer (pH 8.0), 10 µL of DTNB, 10 µL of test-extract solution and 20 µL of acetyl cholinesterase solution were mixed and incubated for 15 min (25°C). 10 mL of acetylthiocholine was added to initiate the reaction. Hydrolysis of acetylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine, at a wavelength of 412 nm (15 min). All the reactions were performed in triplicate in 96-wellmicro-plate. The AChE-inhibitory activity was expressed as inhibition % and was calculated as follows:

% Inhibition = [(Aco – At) / Aco] X 100

Where, Aco is absorbance of the control and At is absorbance of the sample.

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was assessed by the standard method, with slight modifications[31]. Briefly, a volume of 60 µl of sample solution and 50 µl of 0.1 M

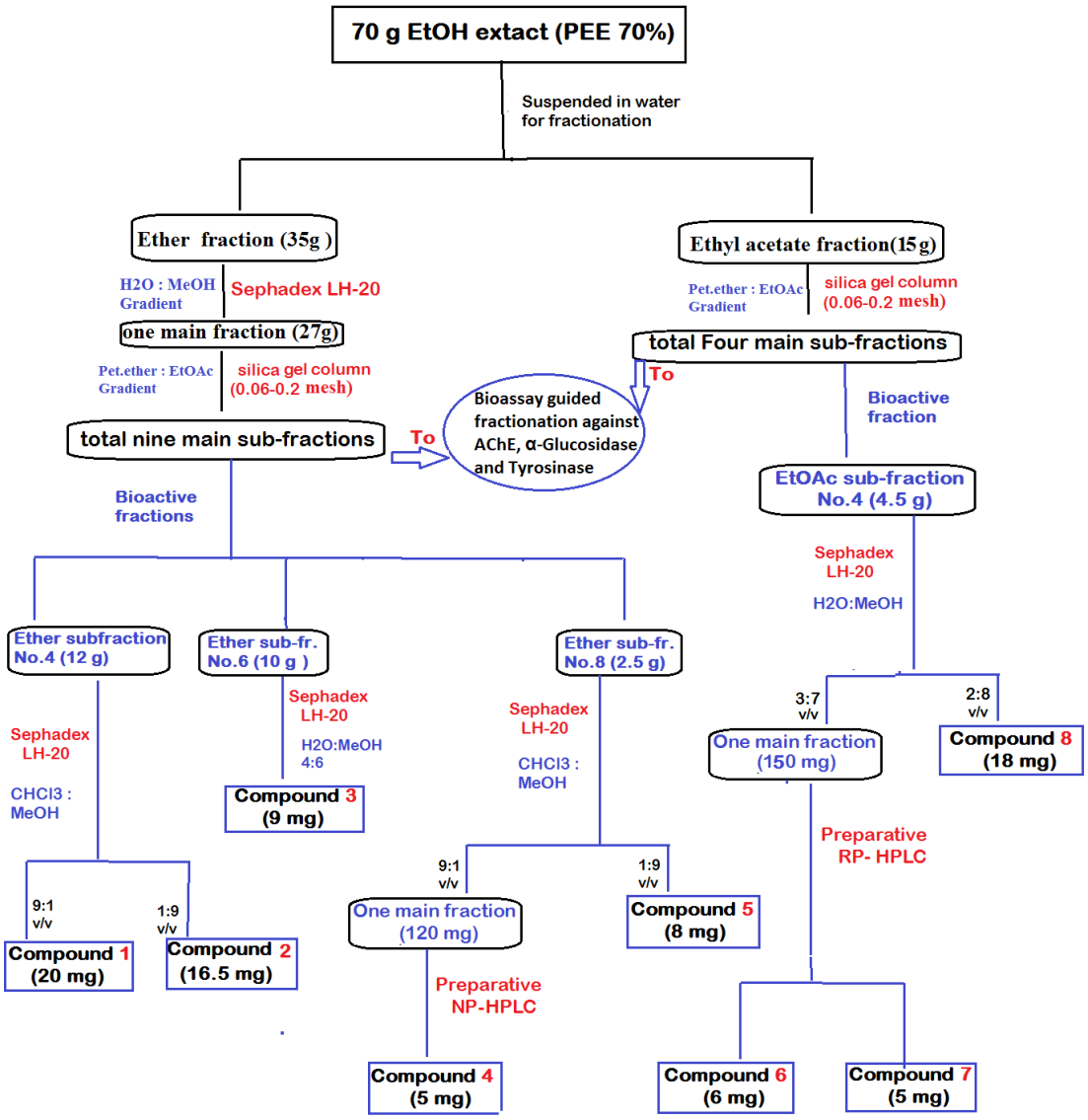

Figure 1: Flowchart of the isolated compounds from Egyptian propolis.

phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U/ml) was incubated in 96 well plates at 37 °C for 20 min. After pre-incubation, 50 µl of 5 mM p-nitrophenyl- α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37 ° C for another 20 min.

Then the reaction was stopped by adding 160 µl of 0.2 M Na_2CO_3 into each well, and absorbance readings (A) were recorded at 405 nm by micro-plate reader and compared to a control which had 60 µl of buffer solution in place of the extract. For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. Commercially available Acarbose was used as a standard and compared with all extracts.

The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

% Inhibition = [(Aco – At) / Aco] X 100

Where, Aco is absorbance of the control and At is absorbance of the sample.

Mushroom Tyrosinase Activity

To evaluate the inhibitory action of methanol extract on tyrosinase, tyrosinase isolated from mushrooms was utilized as described previously with a minor modification[32,33].

In brief, 140 µl 50 mM phosphate buffer (PH 6.8),10 µl of extract (1mg/ml, dissolved in MeOH), 40µl of 1.5mM L-tyrosine solution and 20µlof Mushroom tyrosinase (1500 U/ml) were added to a 96 well microplate. The assay mixture was incubated at 25°C for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm in a microplate reader.

The tyrosinase inhibitory activity was expressed as inhibition % and was

calculated as follows:

% Inhibition = [(Aco – At) / Aco] X 100

Where, Aco is absorbance of the control and At is absorbance of the sample.

RESULTS AND DISCUSSION

Isolation and structure elucidation

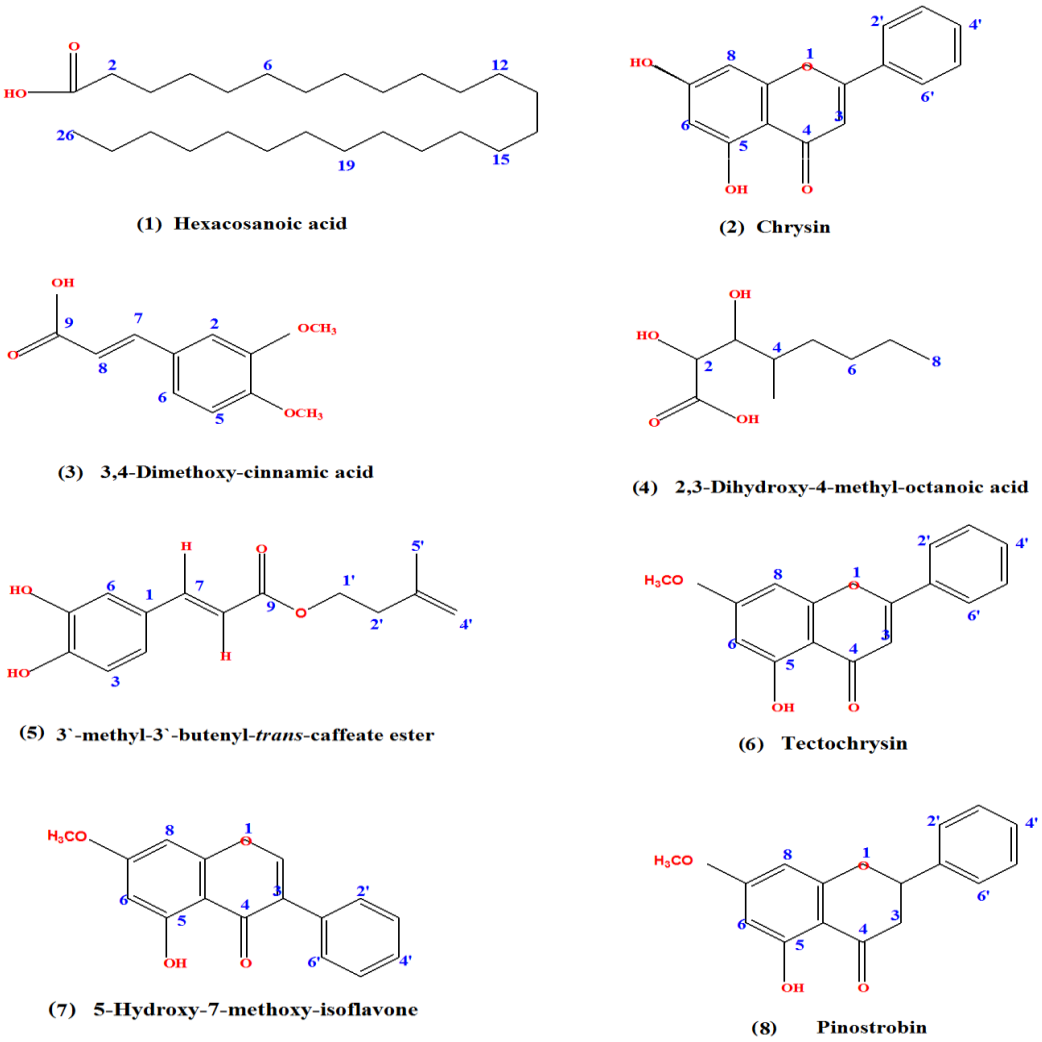

Figure 2: Chemical structures of the isolated compounds from Egyptian propolis.

Eight compounds were isolated from Egyptian Propolis; Hexacosanoic acid; 3,4-di- methoxy-cinnamic acid; 3-methyl-3-butenyl-*trans*-caffeate ester; 2,3-dihydroxy-4-methyl-octanoic acid (which isolated for the first time in Egyptian propolis) together with four flavonoids (chrysin, pinostorbin, tectochrysin and 5-hydroxy-7-methoxy isoflavone (Figure 2).

Acetylcholinesterase (AChE) inhibitory activity

The AChE inhibitory activity of the isolated compounds from Egyptian propolis was evaluated. All flavonoids except chrysin; (pinostorbin, 5-hydroxy-7-methylether isoflavone and tectochrysin) showed variable inhibitory activity (61.7, 42.6 and 38% respectively, Figure 3) compared to the drug used (Distigmine bromide, 75.3%); also 3-methyl-3-butenyl-*trans*-caffeate and 3,4 dimethoxy cinnamic acid showed highly moderate inhibitory activity (57.14 & 56.4% respectively), while hexacosanoic acid had very weak inhibitory activity (4.2%). Drug synergism and antagonism; can explain why a sub-fraction could have biological activity and some of its isolated compounds have no activity and vice versa.

The active site of acetylcholinesterase enzyme was represented by three sub-sites; the esteratic catalytic site (ES), the anionic (AS) and the Peripheral Anionic Site (PAS); compounds bind to this sub-site, suppress the enzyme activity by changing the conformation of the active site[18].

The inhibition process of flavonoids occurs due to the blockage of the entrance to the active site; the carbonyl group in C ring and -OH group in C5 position of the A-ring made hydrogen bonds with PAS sub-site, provide flavonoids with enhanced AChE inhibitor activity. It was reported that 3,4-di-methoxy-cinnamic acid and phenolic compounds having structural similar to caffeic acid showed AChE inhibitor activity.[34,35] Szwajgier stated that "the presence of -OH and -OCH_3 groups elevated anti-AChE activity of cinnamic acid derivatives, depended on that cinnamic acid, exerted no inhibitory activity, while most of its derivatives were active" [36] Our obtained data are in agreement with these findings.

α-Glucosidase inhibitory activity

The inhibitory activity of the isolated compounds were evaluated against α-glucosidase enzyme (Figure 4), tectochrysin showed the highest inhibitory activity (60%), while 5-hydroxy-7-methylether isoflavone and 3,4-dimethoxy cinnamic acid showed very weak inhibitory activity (18% & 10% respectively) in comparison with that of acarbose (49%) .

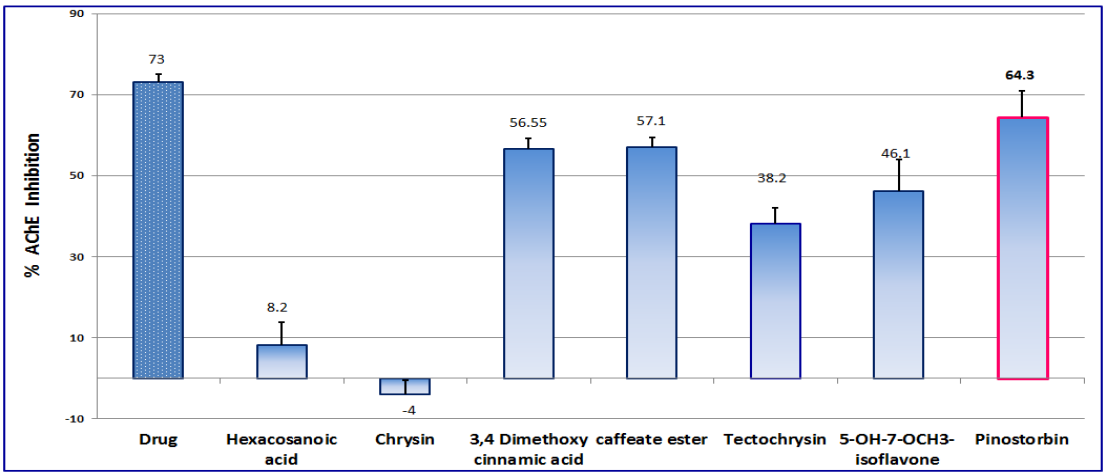

Figure 3: Inhibition activity of propolis compounds against Acetylcholinesterase enzyme. Values are expressed as mean ±SD, n = 3 at a concentration of 100 µM.

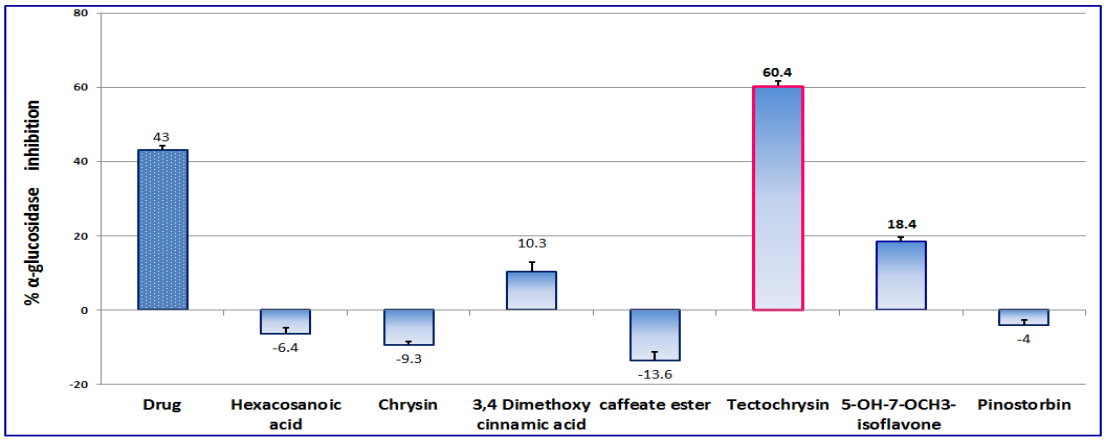

Figure 4: Inhibition activity of propolis compounds against alpha-glucosidase enzyme. Values are expressed as mean ±SD, n = 3 at a concentration of 100 µM.

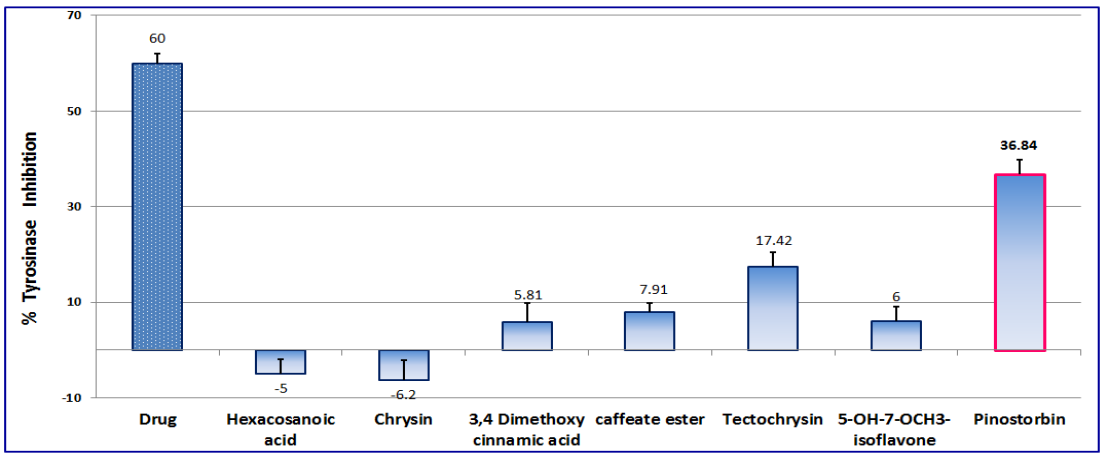

Figure 5: Inhibition activity of propolis compounds against Tyrosinase enzyme. Values are expressed as mean ±SD, n = 3 at a concentration of 100 µM

The others four isolated compounds had no activity. Drug synergism and antagonism; can explain why a sub-fraction could have biological activity and some of its isolated compounds have no activity and vice versa.

One of The main therapeutic approaches in the treatment of diabetes mellitus and its complications is to control the levels of postprandial plasma glucose, which causes hyperglycaemia in type 2 diabetes[37]. Hyperglycemia could be the cause of increasing the level of free radicals, leading to oxidative tissue damage and diabetic complications, which can be controlled by the natural antioxidants polyphenols compounds.

Polyphenols not only reduce oxidative stress but also inhibit carbohydrate hydrolyzing enzymes to prevent hyperglycemia[38]. Many studies proved that the intake of flavonoids is inversely associated with the risk of incident type 2 diabetes[39]. For that, the most effective anti-diabetic compound is preferred to have both hypoglycemic, antioxidant activities and low side effects[40].

Flavonoids with hydroxyl group at C-5, 6 & 7 positions alone have the highest inhibition to α-glucosidase enzyme. The substituent groups of the flavones also affect the enzyme inhibition[41].

Tyrosinase inhibitory activity

Tyrosinase inhibitory activity of the isolated compounds was evaluated. Only pinostorbin showed moderate inhibitory activity (36.3%); while tectochrysin; 3-methyl-3-butenyl-*trans*-caffeate; 5-hydroxy-7-methylether isoflavone and 3,4-dimethoxy cinnamic showed very low inhibitory activity (17.4, 7.9, 6 and 5.8% respectively, in comparison with that of drug (Vit.C, 60%, Figure 5).

Benzoic, cinnamic, methoxy-cinnamic acids and hydroxyl-cinnamoyl derivatives are well known as tyrosinase inhibitors[42,43], many previous studies indicated that methoxy substitutions; 3-methoxy-cinnamic acid and 4-methoxy-cinnamic acid enhanced the inhibition of tyrosinase activity[42]. Kim and his group also reported that 2,4 dihydroxycinnamic ester derivatives significantly reduced tyrosinase activity and melanin synthesis with low cytotoxicity [43].

Flavonoids with hydroxyl group(s) at A and B rings are very important tyrosinase inhibitors by Cu^{2+} chelate formation[45].

The free hydroxyl group or presence of double bond is crucial for anti tyrosinase activity in natural antioxidant[15, 43].

CONCLUSION

In conclusion, these results suggested that propolis polyphenolic/flavonoid components may become attractive and promising treatment for metabolic diseases as Type 2 Diabetes Mellitus " T2DM" ; neurodegenerative diseases as Alzheimer's disease (AD) and acquired skin hyperpigmentation problems but further in-vivo studies are warranted to evaluate the safety and clinical utility of these test components in patients .

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